

# Algicidal Activity of Novel Indigenous Bacterial Strain, *Paracoccus* Sp., Against Harmful Algal Bloom Species, *Karenia mikimotoi*

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## Research Article

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# Abstract

Harmful algal blooms have deleterious effects on aquatic ecosystems and human health. The application of algicidal bacteria is a promising and environmentally friendly method of preventing and eradicating harmful algal blooms. In this study, a screen for algicidal agents against harmful algal blooms was used to identify an algicidal bacterial strain isolated from a *Karenia mikimotoi* culture. Strain O-1 exhibited a strong inhibitory effect on harmful *K. mikimotoi* and was identified as a *Paracoccus* species via 16S rRNA gene sequence analysis. This strain killed *K. mikimotoi* by secreting active algicidal compounds, which were stable at temperatures of -80–121 °C, but these substances were sensitive to strongly acidic conditions. The algicidal properties of strain O-1 against *K. mikimotoi* were cell density- and time-dependent. No significant changes or negative effects were noted for two other Chlorophyta species, which highlighted the specificity of the studied algicidal substance. Finally, single-factor experiments revealed the optimum growth conditions of strain O-1 under different pH and temperature conditions. Strain O-1 therefore has potential as a bio-agent for reducing the biomass of harmful *K. mikimotoi* blooms.

## Introduction

Harmful algal blooms (HABs) frequently occur in eutrophic coastal waters worldwide (Anderson et al. 2002; Hallegraeff 1993). In particular, *Karenia mikimotoi* is a dominant species in HABs in many marine ecosystems. *Karenia mikimotoi* blooms impact marine ecosystem stability by creating an oxygen-depleted environment, producing mucus, and excreting poisonous metabolites, namely hemolytic and ichthyotoxins, thus threatening marine ecological security, marine organisms, the aquaculture and fishing industries, and, ultimately, human health (Lei and Lu 2011; Mooney et al. 2010; O'Boyle et al. 2016; Zheng et al. 2018). These blooms often occur along the coasts of Japan, Europe, and China and are of global concern because of their serious consequences (Kurekin et al. 2014; Aoki et al. 2017; Davidson et al. 2009; Qian et al. 2009).

Various approaches and techniques have been used to control HABs, including chemical control. However, chemical reagents may cause secondary environmental damage (Wang et al. 2017), e.g., pollution by chemical residues, such as copper sulfate, might be very high following the fade of HABs without environmental security (Dethloff et al. 1999). Physical control methods, including ultrasonic and ultraviolet irradiation, have been used to regulate HABs, but these methods are costly and complex, and they often cannot be efficiently applied (Sengco 2009). Biological control methods, on the other hand, are environmentally friendly and potential solutions for controlling HABs. For example, specific groups of heterotrophic bacteria are closely associated with specific algae via mutualistic, commensalistic, or parasitic interactions (Kouzuma and Watanabe 2015), and isolated bacteria or bacteria-derived algicidal compounds have been considered for controlling algal growth (Kim et al. 2008).

The algicidal activity of several bacterial strains has been examined across a broad range of bacterial–algal interactions. For instance, some algicidal bacteria affect specific algal species, whereas others

target a broader range of algae (Kang et al. 2008; Roth et al. 2008). This information can be used to mediate algicidal effects on targeted algal species. Many algicidal bacteria associated with HABs have been isolated and can inhibit harmful algae through various modes of activity, such as direct cell-to-cell contact or algicidal compounds. For example, cell-free filtrate from cultures of the bacterium *Micrococcus luteus* strain SY-13 caused cell lysis and motility loss in the dinoflagellate *Cochlodinium polykrikoides* (Kim et al. 2008). In contrast, the algicidal activity of *Flavobacteriaceae* strains require direct contact with the dinoflagellate *Karenia brevis* (Roth et al. 2007). Many efforts have been made to identify bioactive algicidal compounds produced by bacteria and their capability to kill HABs. For example, anti-algal polyunsaturated fatty acids from the seaweed *Ulva fasciata* can inhibit the microalga *Heterosigma akashiwo* (Alamsjah et al. 2005).

In this study, we screened a novel bacterial strain, O-1, that has shown high algicidal activity against the harmful alga *K. mikimotoi* and identified it based on 16S rDNA sequencing and phylogenetic analysis. The responses of *K. mikimotoi* to the algicidal activity of strain O-1 at different dosages were also investigated, and the quantity of this strain was investigated under varying temperature and pH conditions. The main objectives of this study were to examine the mode of action of strain O-1 against *K. mikimotoi*, to explore the specificity of the algicidal activity and characteristics of extracellular algicidal compounds, to reveal the activity of algicidal bacteria against algal cells, and to improve understanding of the bio-control mechanism of algicidal bacteria against HABs.

## Materials And Methods

### *Karenia mikimotoi* culture

*Karenia mikimotoi* used in this study was purchased from the Laboratory of Microalgae Research, Ocean University of China, Qingdao, Shandong Province, China. Algal cells were cultured in sterile f/2 medium prepared with natural seawater in conical flasks (Lanahan et al. 2013) and incubated at a constant temperature of 25 °C, illumination of 3000  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , and 12:12 h light:dark cycle.

### Determination of algicidal activity of strain O-1 against *K. mikimotoi*

A bacterial strain previously isolated from a *K. mikimotoi* culture, namely O-1, was used. To screen for an isolated pure bacterial strain with algicidal activity against *K. mikimotoi*, strain O-1 was incubated in 2216E medium (5 g L<sup>-1</sup> peptone, 1 g L<sup>-1</sup> yeast extract, and 0.01 g L<sup>-1</sup> ferric phosphorous; pH 7.6–7.8 in seawater), cultured, and shaken at 120 rpm at a constant temperature of 30°C for 48 h. Then, 1, 3, and 5 mL bacterial solutions (with volume ratios of 1, 3, and 5%, respectively) were each inoculated into a 100 mL flask containing 50 mL sterile f/2 medium and 50 mL exponential growing algal cultures with an initial cell concentration of  $3.0 \times 10^4$  cells mL<sup>-1</sup>. The mixed bacterium–*K. mikimotoi* cultures were all incubated under *K. mikimotoi* culture conditions as described above for 5 d. As a control, sterile 2216E medium without bacteria was added to the *K. mikimotoi* culture. Different morphologies of *K. mikimotoi*

cells were observed every 24 h for 5 d with an inverted microscope. After the algal cells were fixed with Lugol's iodine, the cell number of all samples were counted using a hemocytometer. The following equation was used to calculate the algicidal activity of strain O-1: Algicidal activity (%) =  $(1 - Tt/Ct) \times 100\%$ , where  $T$  and  $C$  are the concentrations of *K. mikimotoi* cells in the treatment and control groups, respectively, and  $t$  is the incubation time. All experiments were conducted in triplicate.

## Identification of strain O-1

The 16S rRNA gene of strain O-1 was identified via molecular methods according to Zheng et al. (2018). PCR amplification and sequencing of O-1 16S rRNA were also performed according to Zheng et al. (2018) using amplification primers 27F and 1492R (Winker and Woese 1991). The PCR reaction mixture consisted of 10.5  $\mu$ L sterile ddH<sub>2</sub>O, 4  $\mu$ L 5 $\times$ TransStart®FastPfu Buffer (TransGen Biotech, China), 2  $\mu$ L 2.5 mM dNTP, 0.5  $\mu$ L TransStart®FastPfu DNA Polymerase, 0.5  $\mu$ L forward primer (10  $\mu$ M), 0.5  $\mu$ L reverse primer (10  $\mu$ M), and 2  $\mu$ L sample DNA. Thermocycling involved initial denaturation at 95°C for 10 min, followed by 30 cycles of 30 s at 95°C and annealing at 55°C for 30 s and 72°C for 1 min. The PCR amplicons were sequenced on an ABI 377 DNA sequencing system. The closest genus relatives of each isolate were identified by comparing the 16S rRNA sequences with sequences deposited in the National Center for Biotechnology Information GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST>) using the Basic Local Alignment Search Tool. A phylogenetic tree was constructed using the neighbor-joining method in MEGA 5.0 software (Yang et al. 2013). Sequences of the 16S rRNA gene were submitted to GeneBank ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) with the accession number MG457257.

## Testing of algicidal activity of strain O-1 against other algal species

*Platymonas helgolandica* and *Chlorella* sp. used here were donated by the Laboratory of Microalgae Research, Ocean University of China. To test the algicidal activity of strain O-1 against athecate (naked) dinoflagellate (*K. mikimotoi*) and two Chlorophyta species, the isolate was first cultured in 100 mL 2216E medium and grown to the stationary phase at 25 °C (within 48 h). Subsequently, 3 mL culture was inoculated into three 100 mL flasks, each containing 50 mL sterile f/2 medium and 50 mL of each of the three exponentially growing algal cultures. All experiments were carried out in triplicate. As a control, sterile 2216E medium without bacteria was added to each of the three algal cultures. The algal cells were counted every 24 h for 96 h. The algicidal activity was determined according to the following equation: Algicidal activity (%) =  $(1 - Nt/Nc) \times 100$ , where  $Nc$  is the number of algal cells in the control group, and  $Nt$  is the number of algal cells in the treatment group.

## Determination of algicidal mode of strain O-1 against *K. mikimotoi*

To determine the mode responsible for the algicidal activity of strain O-1, different treatments, namely bacterial culture, cell-free filtrate, and bacterial cells were used: (1) a 3% of bacterial culture was added to a *K. mikimotoi* culture, (2) a 3% bacterial culture was centrifuged at 11 000 $\times g$  for 20 min at 4 °C, and the

supernatant was filtered through a 0.22 µm membrane filter (Merck Millipore, Darmstadt, Germany), and (3) the remaining sediment was washed twice, and the cells were resuspended in the same volume (3%) of f/2 medium and labeled as O-1 cells. 3% of sterile 2216 medium were added to the algal culture as a control. All the conditions were as described above in “determination of algicidal activity of strain O-1” (for a treatment time of 48 h).

## Characterization of algicidal compounds in strain O-1

To determine the thermal stability of the algicidal compounds produced by strain O-1, Three separate cell-free filtrates (the same filtrates as were used to determine the algicidal mode) were submerged in boiling water at 80°C for 20 min, frozen at -80°C for 24 h, and autoclaved at 121°C for 20 min, respectively. The 3 mL filtrate in each of these treatments was allowed to return to 20°C before adding it to a 100 mL *K. mikimotoi* culture at a 3% final volume to determine the algicidal activity. The acidic/alkaline stability of the extracellular algicidal compounds was tested by adjusting the pH of the strain O-1 cell-free filtrates at different pH values (i.e., 2.0, 4.0, 10.0, and 12.0). All experiments were conducted in triplicate. The algicidal activity was estimated as indicated above in “determination of algicidal activity of strain O-1”.

## Determination of quantity of strain O-1 under different conditions

The quantity (number of strain cells) of strain O-1 was determined under different temperature and pH conditions, and two treatments were implemented: (1) Strain O-1 was incubated in 2216E medium and shaken at 120 rpm at 20, 25, 30, and 35°C for 48 h, respectively, and (2) the pH of the bacterial culture was adjusted to 6.0, 6.5, 7.0, 7.5, and 8.5, using citric acid/sodium citrate (pH 3.0–6.5) and Na<sub>2</sub>HPO<sub>4</sub>/citric acid (pH 7.0–8.5) buffers, incubated in 2216E medium, and shaken at 120 rpm at 30°C for 48 h. The number of O-1 cells was counted every 24 h by measuring the optical density at a wavelength of 600 nm (OD<sub>600</sub>) via ultraviolet spectrophotometry. All experiments were conducted in triplicate.

## Data analysis

To analyze the differences between means, one-way analysis of variance was carried out in SPSS 17.0 (SPSS Inc., Chicago, IL, USA). All data were presented as a mean with standard error and were analyzed using one-way analysis of variance followed by the least significant difference test, with  $P < 0.05$  (Origin 8.5 for Windows; Redmond, WA, USA).

## Results

### Identification of isolated bacteria

A total of 14 bacterial strains previously were isolated from a *K. mikimotoi* culture. Among these strains, bacterium O-1 exhibited the highest algicidal activity (> 85%) against *K. mikimotoi*. The 16S rRNA gene sequence of this bacterium was identified as a *Paracoccus* sequence. The phylogenetic tree of strain O-1

is shown in Fig. 1. According to the 16S rRNA gene sequence and phylogenetic analysis, strain O-1 is a *Paracoccus* species (Alphaproteobacteria).

## Algicidal activity of strain O-1 against *K. mikimotoi*

Figure 2 shows that the algicidal activity of strain O-1 against *K. mikimotoi* varied under different dosages of bacterial culture over a period of 5 d. The 1% dosage slightly stimulated the growth of *K. mikimotoi*, no algicidal effects were observed ( $P > 0.05$ ). Algal growth inhibition was observed with the 3% dosage, and *K. mikimotoi* cell density was as low as  $0.41 \times 10^4$  cells  $\text{mL}^{-1}$  (amount to 3.4% of control group) after 5 d, whereas that of the control was  $1.2 \times 10^5$  cells  $\text{mL}^{-1}$ . The 5% dosage showed the strongest algicidal effect, and no intact algal cells were observed under the microscope after 5 d. Therefore, with the extension of time, high dosages (3 and 5%;  $P < 0.05$ ) of the O-1 culture were more effective than the low dosage (1%,  $P > 0.05$ ) at inhibiting *K. mikimotoi* growth. The algicidal activity against *K. mikimotoi* growth was concentration- and time-dependent.

## Effect of strain O-1 on different algal species

To assess the specificity of algicidal activity displayed by strain O-1 a further 2 species of Chlorophyta were tested for sensitivity (Fig. 3). Strain O-1 had the greatest inhibitory activity on *K. mikimotoi* across all test times ( $> 75\%$  activity;  $P < 0.01$ ). However, no algicidal effect was observed in either of the tested Chlorophyta species (Fig. 3), i.e., strain O-1 did not significantly affect non-HAB *Chlorella* species ( $< 15\%$  activity) or *Platymonas helgolandica* ( $< 0\%$  activity). Strain O-1 therefore exhibited species-specific algicidal activity.

## Algicidal mode of strain O-1 against *K. mikimotoi*

The algicidal mode of strain O-1 against *K. mikimotoi* is shown in Fig. 4. Different treatments, namely bacterial culture, cell-free filtrate, and bacterial cells were co-cultured with *K. mikimotoi*. The strain O-1 culture showed strong algicidal activity after 48 h, with an algicidal rate of 94%. The addition of cell-free filtrate of strain O-1 reduced algal cell density, and the algicidal activity against *K. mikimotoi* was 92.1%. Significant algicidal activities in the bacterial culture and cell-free filtrate treatments were observed ( $P < 0.05$ ). However, the O-1 cells had no significant inhibitory effect on *K. mikimotoi* growth and had an algicidal activity of only 4.7% ( $P > 0.05$ ). These results imply that strain O-1 expressed algicidal activity via an indirect mode.

## Thermal and pH stability of algicidal compounds in strain O-1

To determine the algicidal compounds characteristics, the thermal and pH stability of algicidal compounds in strain O-1 were investigated (Fig. 5). No significant differences existed in the activity of the algicidal compounds after freezing, boiling, or autoclaving, i.e., exposure to temperatures of -80, 80, or 121°C, respectively, for 48 h ( $P > 0.05$ ), and the algicidal activities remained high and stable at different temperatures (Fig. 5A). On the other hand, pH affected the activity of the algicidal compounds (Fig. 5B).

No algicidal activity was detected after exposure to very acidic conditions (pH 2;  $P < 0.01$ ), but the algicidal activity at pH 4, 10, or 12 did not differ significantly ( $P > 0.05$ ).

## Quantity of strain O-1 under different conditions

Single-factor experiments were conducted to optimize the high cell-density conditions of strain O-1. Temperature significantly affected the growth of strain O-1 (Fig. 6A). The highest bacterial concentration occurred at 30°C after 48 h (OD<sub>600</sub>: 1.146), and this was deemed the optimal temperature for the growth of strain O-1, whereas the lowest bacterial concentration (OD<sub>600</sub>: 0.817) was observed at 20°C. The bacterial concentrations of strain O-1 at 25 and 35°C were also lower than that at 30°C after 48 h. Figure 6B illustrates the cell density of strain O-1 at 30°C at varying pH levels. After 48 h, the mean OD<sub>600</sub> values of strain O-1 were 1.211, 1.304, 0.931, 0.404, and 0.679 at pH 6.0, 6.5, 7.0, 7.5, and 8.5, respectively. The highest bacterial concentration was therefore observed when strain O-1 was incubated under weakly acidic conditions (pH 6.5).

## Discussion

HABs are serious environmental hazards in many coastal areas because of the growing human population size and increasing industrialization, and they threaten co-occurring aquatic organisms by producing toxins. Algal–bacterial interactions are key to regulating and decreasing *K. mikimotoi* blooms. In this study, a novel indigenous bacterial strain of *Paracoccus* sp. (O-1) with potent algicidal activity was isolated from a *K. mikimotoi* culture. After algal cells were treated with strain O-1 with the 3 and 5% dosage, they were significantly inhibited, and algicidal activity of this bacterial strain reached 90% in 72 h, suggesting that this strain effectively killed algal cells. While reports on the application of *Paracoccus* to control HABs are limited, the algicidal activity of this species may play an important role in bacterial–algal interactions and natural attenuation of HABs.

The O-1 culture used in this study exhibited strong algicidal activity (> 90%), but the specific mechanism remains unfamiliar. Therefore, the mode of algicidal action should be identified. Algicidal mechanisms can be classified into two general modes, namely a direct mode, where bacterial cells must be in contact with algal cells for successful cell lysis, or an indirect mode, where an extracellular metabolite produced by algicidal bacteria is responsible for the algicidal activity (Mayali and Azam 2004; Pokrzywinski et al. 2012). The O-1 algicidal mode was found to be indirect, as the algicidal effects were likely expressed via the production of extracellular algicidal compounds, as opposed to direct cell–cell contact. These results agree with those of another study, which found that *Deinococcus* sp. strain Y35 exhibited a strong algicidal effect on the toxic dinoflagellate *Alexandrium tamarense* by producing extracellular pigments (Li et al. 2015).

To characterize the algicidal activity of the extracellular compounds of strain O-1, our results suggest that these algicidal compounds tolerated a relatively wide range of temperatures and pH conditions, but the algicidal activity was diminished under strong acidic conditions, implying that these extracellular substances were thermally stable but sensitive to acidic conditions. Other studies have reported the

temperature and acidic/alkaline stability of bacterial algicides. An algicidal compound produced by *Pseudomonas aeruginosa* showed no change in algicidal activity after being autoclaved at 121°C for 15 min and stored at 4°C for 3 months (Dakhama et al. 1993). In contrast, Lee et al. (2000) showed that an algicidal serine protease released by *Pseudoalteromonas* sp. strain A28 had diminished activity after being heated for 15 min at 100°C (Lee et al. 2000). Li et al. (2016) also noted that algicidal substances produced by *Exiguobacterium* sp. strain h10 exhibited high thermal stability and pH instability (Li et al. 2016).

Bacterial compounds with algicidal activity can be classified into three main types: proteins or proteases (Lee et al. 2002), pigments (Li et al. 2015), and surfactants (Wang et al. 2005). The algicidal compound produced by strain O-1 in this study has yet to be identified, but its thermal stability and pH instability suggest that it is not a nucleic acid or protein. This algicidal compound is likely to be a pigment or glycolipid (Kaytee et al. 2012). This result agrees with those of another study, Malimas et al. (2008) identified the pigments produced by *Gluconobacter sphaericus* (Malimas et al. 2008). The algicidal compound produced by strain O-1 may be a novel algicide for *K. mikimotoi* blooms and should be identified and further characterized in future studies.

To determine the specificity of the algicidal activity of strain O-1, its effects on two other Chlorophyta species were tested. Strain O-1 had a strong inhibitory and lethal effect on *K. mikimotoi*, whereas it was ineffective on the other two tested species. The algicidal activity of strain O-1 was therefore exhibited species-specific. These results conclude that fibrous cell wall of chlorophytes may make them resistant to the “non-nucleic acid or non-protein” metabolite. Similarly, Doucette et al. (1999) revealed that strain N-3 had a relatively specific inhibitory effect on the dinoflagellate *Gymnodinium nagasakiense* (Doucette et al. 1999). Algicidal bacteria that have been described may aid the development of technologies for mitigating HABs.

The algicidal activity of strain O-1 against *K. mikimotoi* depended on the concentration of the bacterial culture and treatment time. Compared to the control group, the 1% bacterial dose stimulated the growth of *K. mikimotoi* after 5 d (Fig. 2). This may be related to hormesis (Stebbin 1982), as *K. mikimotoi* cells exhibited some resistance to the adverse effects of the O-1 algicide. At bacterial doses of 3 and 5%, the algicidal effect was complete after 5 d. The algicidal activity was somewhat correlated to the bacterial cell density (Zhang et al. 2016). Increased algicidal activity may have been due to increased algicidal compound production per cell with increased bacterial growth under favorable conditions.

To optimize the culture conditions of O-1 cells, a single-factor design experiment was conducted to determine the optimal thermal and pH conditions. The optimal conditions for this bacterium occurred at 30 °C and pH 6.5. Ma et al. (2015) investigated the influence of environmental factors, including temperature, illumination intensity, and pH, on the growth of two algicidal bacteria using the Plackett–Burman design (Ma et al. 2015). Our results suggested that environmental factors (pH and temperature) may play essential roles in the quantity of strain O-1, thereby these factors significantly affected the

algicidal activity of the algicidal bacterial strain, which was consistent with the results of a previous study (Yoon et al. 2011).

In conclusion, this study showed that the screening of novel *Paracoccus* sp. algicide, is beneficial for increasing knowledge and understanding of bacteria with bioactive potential in the marine environment. Strain O-1 has the potential to be an algicidal bacterium against HABs caused by *K. mikimotoi* in future.

## Declarations

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### Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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## Figures

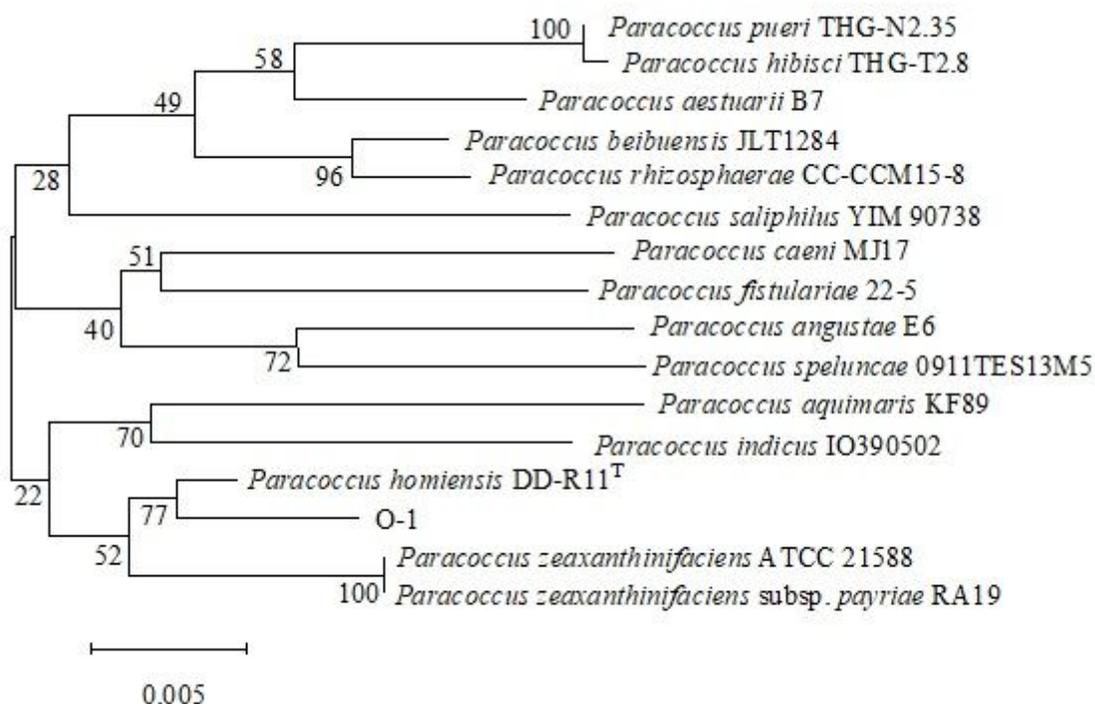


Figure 1

Phylogenetic tree based on bacterial 16S rRNA gene sequence of isolated bacterial strain O-1 and closely related members. Scale bar represents 0.005 nucleotide substitutions per position.

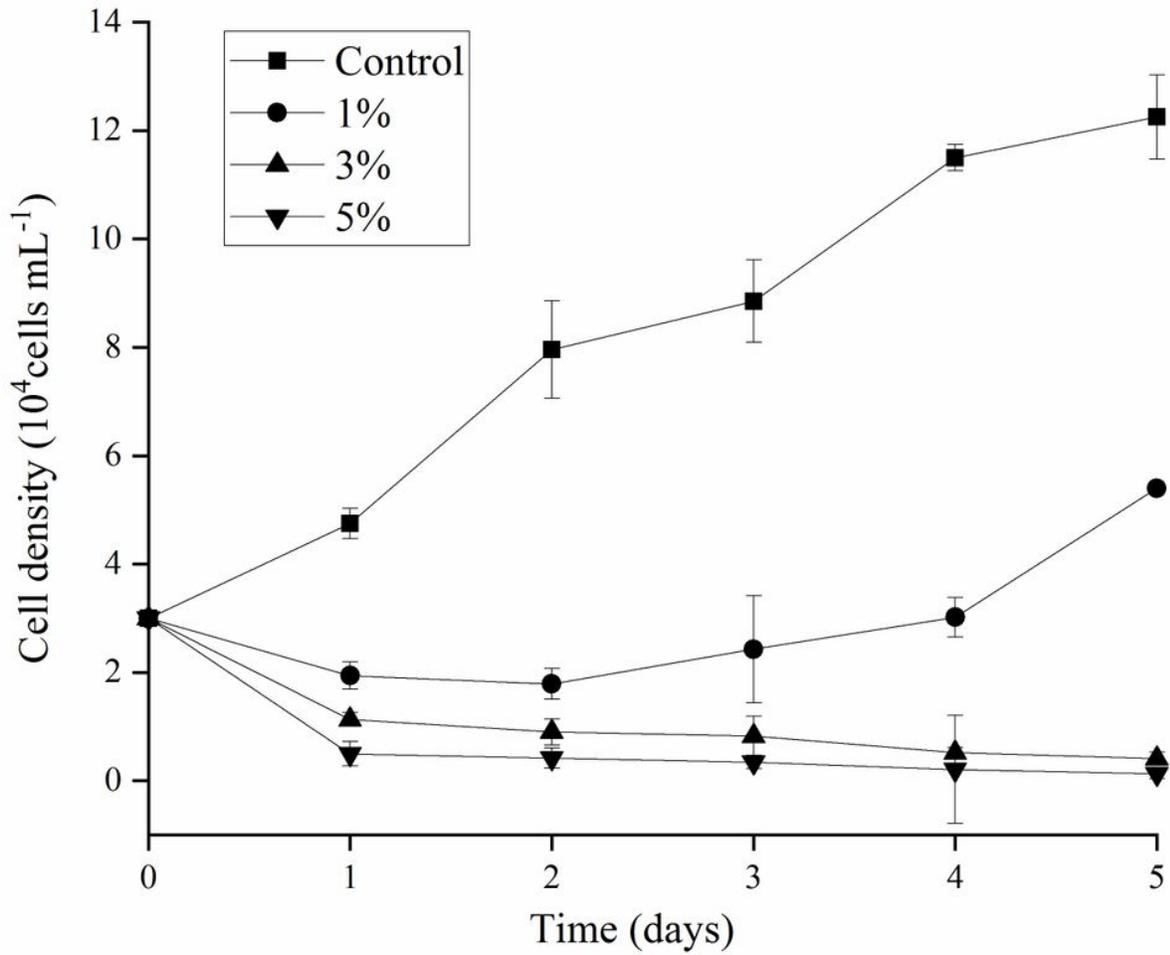
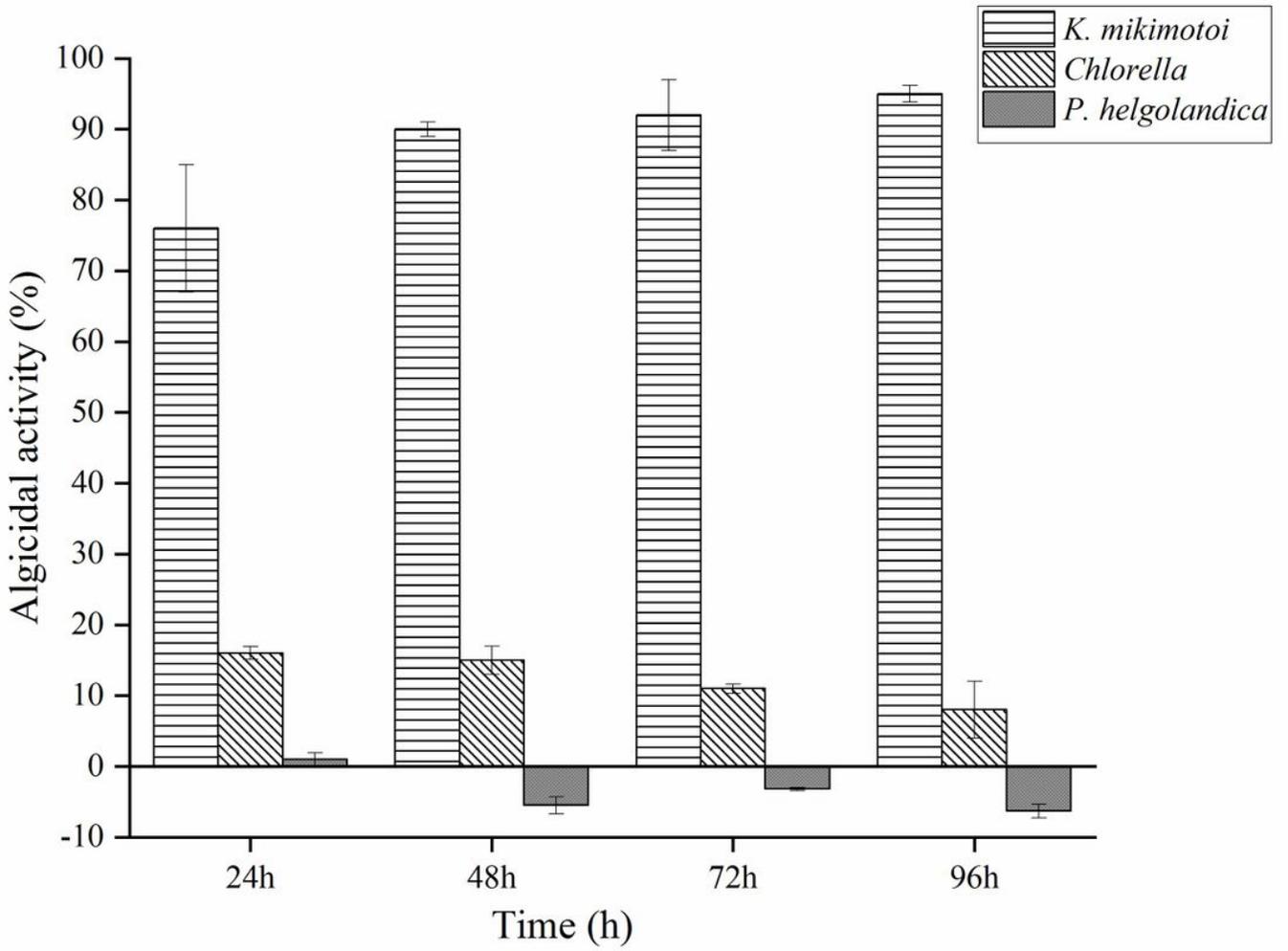


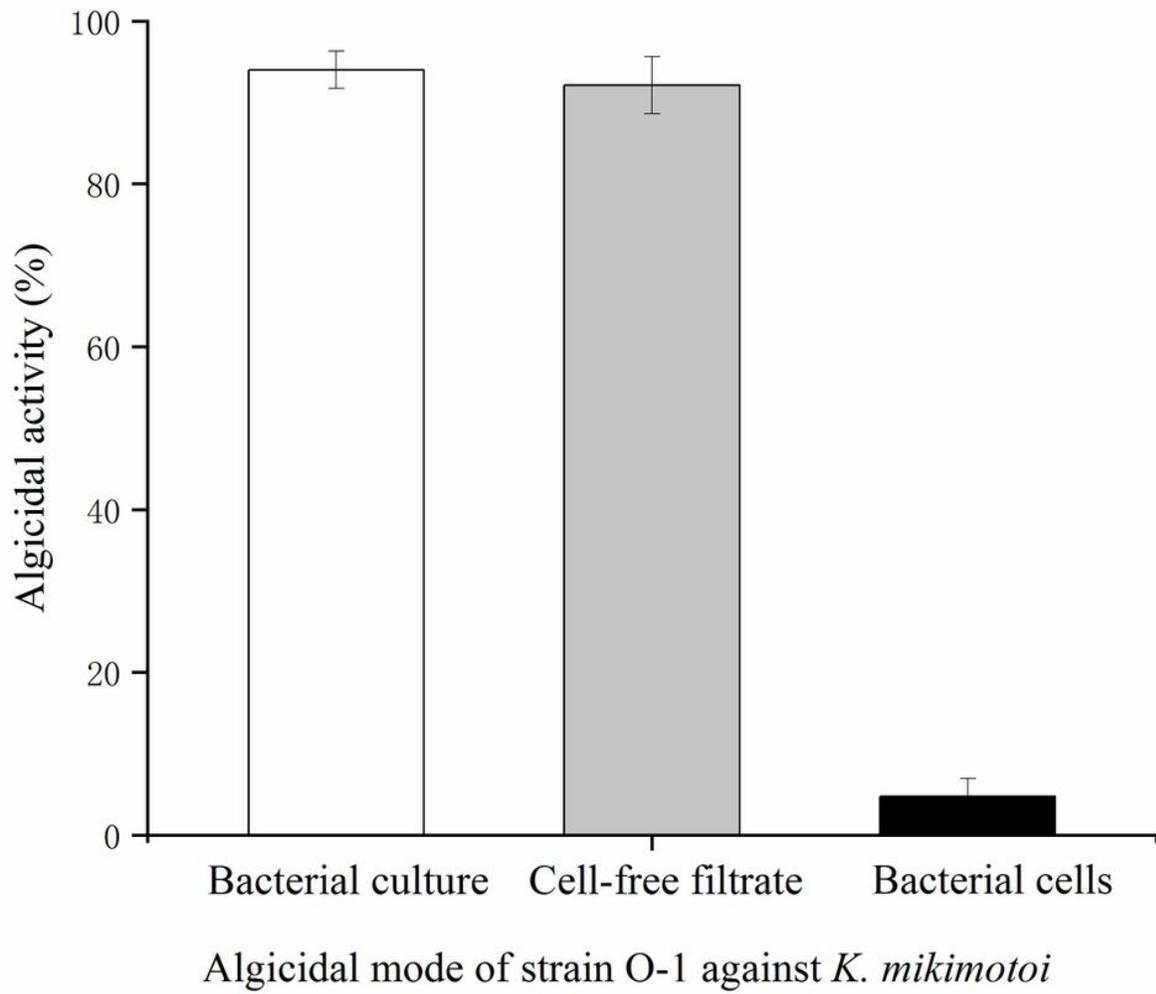
Figure 2

Algicidal activities of different dosages of bacterial strain O-1 against *Karenia mikimotoi*.



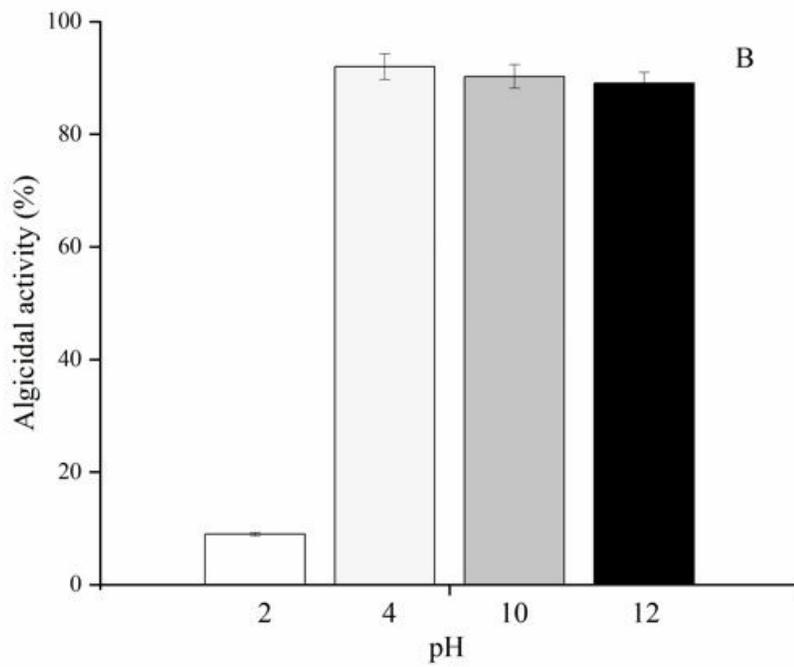
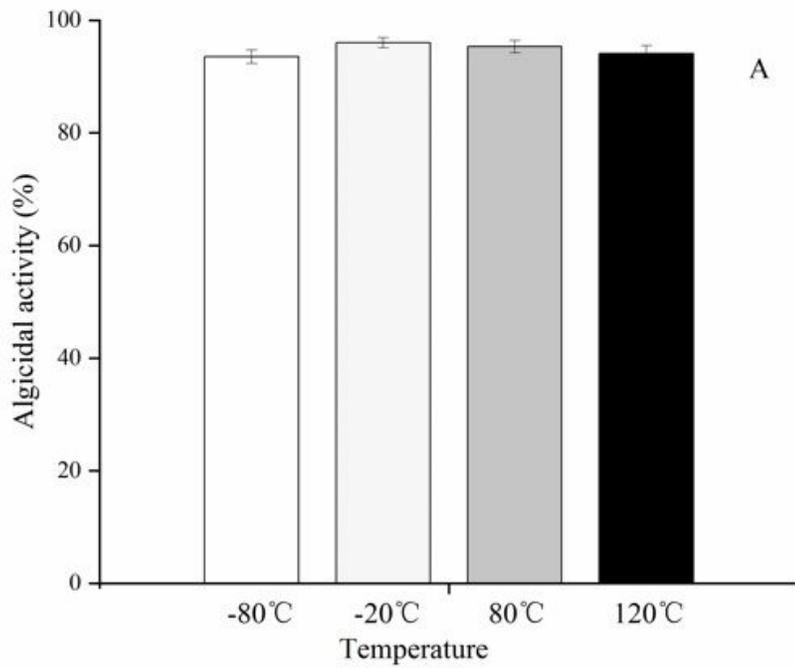
**Figure 3**

Algicidal effects of bacterial strain O-1 on different algal species (*Karenia mikimotoi*, *Chlorella*, and *Platymonas helgolandica*).



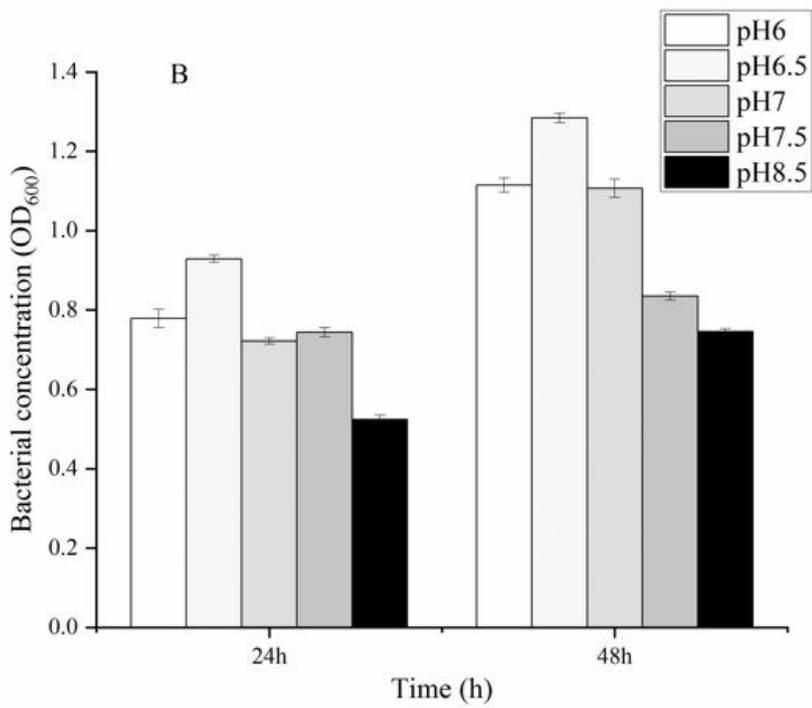
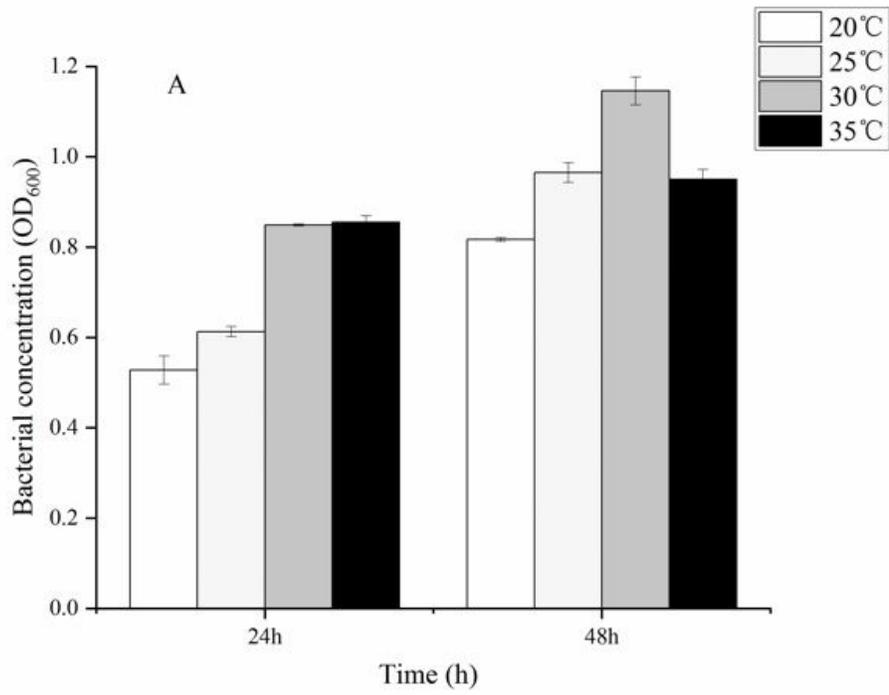
**Figure 4**

Algicidal activities against *Karenia mikimotoi* of different strain O-1 treatment groups, namely bacterial culture, cell-free filtrate, and bacterial cells.



**Figure 5**

Characteristics of algicidal compounds in strain O-1, namely (A) thermal stability and (B) pH stability of extracellular algicidal compounds.



**Figure 6**

Concentrations of strain O-1 under different (A) temperature and (B) pH conditions.